THE EFFECTS OF TUALANG HONEY ON NOCICEPTIVE RESPONSES IN THE THALAMUS OF PRENATALLY STRESSED MALE RATS OFFSPRING

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LIST OF ABBREVIATIONS

11βHSD2	11-β-hydroxysteroid dehydrogenase type-2
4-HNE	4-hydroxynonenal
4PL	Four Parameter Logistic
5-HT	5-hydroxytryptamine or serotonin
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactive disorder
ANOVA	Analysis of variance
ARASC	Animal Research and Service Centre
ATP	Adenosine triphosphate
CAT	Catalase
CI	Confidence interval
CRF	Corticotrophin releasing factor
CRH	Corticotrophin-releasing hormones
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FPU	Foetal-placental unit
GABA	Gamma-Aminobutyric acid
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
HCEP	Epithelial progenitor cell
HPA	Hypothalamic-pituitary-adrenal

IASP	International Association for the Study of Pain
MDA	Malondialdehyde
Na_2HPO_4	Di-sodium hydrogen phosphate anhydrous
$Na_2HPO_4.7H_2O$	Di-sodium hydrogen phosphate heptahydrate
NaCl	Sodium chloride
$NaH_2PO_4.H_2O$	Sodium Dihydrogen Phosphate Monohydrate
NaOH	Sodium hydroxide
NF-B	Nuclear factor kappa B
NMDA	N-methyl-D-aspartate receptors
NR1	NMDA Receptor 1
NR2	NMDA Receptor 2
mRNA	Messenger ribonucleic acid
PAG	Periaqueductal grey
R^2	Correlation coefficient
ROS	Reactive oxygen species
SEM	Standard error mean
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
VPL	Ventral posterolateral
VPM	Ventral posteromedial
VL	Ventral lateral

KESAN MADU TUALANG TERHADAP TINDAK BALAS NOSISEPTIF DALAM TALAMUS PADA ANAK TIKUS JANTAN YANG TELAH DIDEDAHKAN TEKANAN SEMASA DALAM PRANATAL

ABSTRAK

Pelbagai rumusan mekanisme perubahan tindak balas terhadap nosiseptif anak yang terdedah kepada tekanan pranatal; namun tidak ada satu kajian yang menyelidiki peranan talamus dalam modulasi nosiseptif. Tujuan pertama kajian ini adalah untuk mengkaji kesan tekanan pranatal terhadap tingkah laku nosiseptif, perubahan morfologi, paras parameter tekanan oksidatif dan reseptor NMDA dalam talamus pada anak tikus jantan yang telah diberi tekanan semasa dalam pranatal. Tujuan kedua adalah untuk menentukan kesan madu Tualang terhadap parameter yang dikaji. Tiga puluh tiga ekor tikus Sprague Dawley yang bunting telah dimasukkan secara rambang ke dalam kumpulan kawalan, tekanan dan tekanan yang dirawat dengan madu Tualang. Madu Tualang (1.2g / kg berat badan / hari) atau air suling telah diberikan secara oral kepada tikus bunting pada kumpulan yang sepadan. Tekanan kekangan telah diberikan kepada kumpulan tekanan dan kumpulan yang dirawat dari hari ke 11 kebuntingan hingga melahirkan anak. Najis ibu telah dikumpulkan (pada hari ke 11 dan 21) untuk mengukur paras kortikosteron. Selepas kelahiran, anak tikus dewasa didedahkan kepada ujian tingkah laku nosiseptif (n = 7 setiap kumpulan). Dalam ujian formalin, skor tingkah laku nosiseptif mereka telah dicatatkan. Tikus telah dikorbankan dua jam selepas suntikan formalin. Selepas itu, talamus anak tikus jantan dikeluarkan untuk menentukan paras parameter tekanan oksidatif dan reseptor NMDA manakala pewarnaan Nissl dilakukan untuk mengesan perubahan morfologi dalam talamus. Semua data dianalisis dengan menggunakan

SPSS, versi 22. Skor tingkah laku nosiseptif dianalisis dengan menggunakan analisis varians pengiraan berulang (ANOVA) dengan pembetulan Bonferroni dan data yang selebihnya dianalisis dengan menggunakan ANOVA satu hala. Hasil kajian ini menunjukkan penurunan yang ketara dalam skor tingkah laku nosiseptif (P <0.05), paras malondialdehida yang rendah dengan paras katalase (P <0.05), superoxida dismutase (P <0.05) dan glutation (P <0.001) yang lebih tinggi dalam kumpulan tekanan yang dirawat dengan madu Tualang berbanding dengan kumpulan tekanan. Pemerhatian histologi menunjukkan pengurangan neuron Nissl positif yang jelas (P <0.05) dalam nukleus ventral posteralateral talamus di bahagian sebelah kiri pada kumpulan tekanan berbanding kumpulan lain. Daripada kajian ini, ia dapat disimpulkan bahawa tekanan prenatal menyebabkan perubahan pada tingkah laku nosiseptif, parameter tekanan oksidatif, morfologi dan paras reseptor NMDA dalam talamus anak tikus jantan. Selain itu, pemberian madu Tualang semasa tekanan pranatal dikaitkan dengan penambahbaikan dalam tingkah laku nosiseptif, parameter tekanan oksidatif, dan morfologi dalam talamus anak tikus jantan. Walau bagaimanapun, pemberian madu Tualang tidak menyebabkan penurunan yang ketara pada paras reseptor NMDA. Kajian ini memberikan pengetahuan baru mengenai kemungkinan mekanisme antioksidan madu Tualang dalam mengubah tindak balas nosiseptif dalam talamus anak tikus jantan yang telah diberi tekanan semasa dalam pranatal.

THE EFFECTS OF TUALANG HONEY ON NOCICEPTIVE RESPONSES IN THE THALAMUS OF PRENATALLY STRESSED MALE RATS OFFSPRING

ABSTRACT

Various mechanisms have been postulated to contribute to alteration of nociceptive responses in offspring exposed to prenatal stress; however not a single study has investigated the possible role of thalamus in the nociceptive modulation. The first aim of this study was to investigate the effects of prenatal stress on nociceptive behaviour, morphological changes, level of oxidative stress parameters and NMDA receptors in the thalamus of prenatally stressed male rats offspring. The second aim was to determine the effects of Tualang honey on these parameters. Thirty-three Sprague Dawley pregnant rats were randomised into control, stress and stress-treated with Tualang honey groups. Oral administration of Tualang honey (1.2g/kg body weight/day) or distilled water was given to the corresponding pregnant dams. Restraint stress was applied to the stress and stress-treated groups from day 11 of pregnancy until delivery. Maternal faeces were collected (on day 11 and 21) to measure the level of corticosterone. After parturition, male rats offspring were subjected for nociceptive behavioural testing (n=7 per group). In formalin test, their nociceptive behavior score was recorded. The rats were sacrificed two hours post formalin injection. After that, thalamus of the male rats offspring was removed to determine the level of oxidative stress parameters and NMDA receptors while Nissl staining was performed to detect the morphological changes in the thalamus. All data were analysed by using SPSS, version 22. Nociceptive behaviour score was analysed

by using repeated measures analysis of variance (ANOVA) with Bonferroni correction and the remaining data were analysed by using one-way ANOVA. The results of this study demonstrated a significant decrease in nociceptive behaviour score (P<0.05), lower malondialdehyde level with higher catalase, superoxide dismutase activities (P<0.05) and glutathione (P<0.001) in stress-treated with Tualang honey group compared to stress group. Histological observations demonstrated a significant (P<0.05) reduction of Nissl-positive neurons in the left ventral posterolateral thalamic nuclei of the stress group compared to other groups. From this study, it can be concluded that prenatal stress was associated with alteration in nociceptive behavior, oxidative stress parameters, morphology and level of NMDA receptors in the thalamus of adult rats offspring. In addition, Tualang honey administration was associated with improvement in nociceptive behaviour, oxidative stress parameters and morphology in the thalamus of male rats offspring. However, Tualang honey administration did not significantly reduce the NMDA receptors level. The present study provides new knowledge regarding possible mechanisms of antioxidant property of Tualang honey in modulating the nociceptive responses in the thalamus of prenatally stressed male rats offspring.

CHAPTER 1

INTRODUCTION

1.1 Background of study

It is estimated that up to 20% of pregnant women experience anxiety and depression (Barlow *et al.*, 2014). Exposure to physiological stress during pregnancy has been associated with pregnancy complications and disorder in offspring's cognitive, behaviour and physical development. Variations in prenatal environment and condition can affect the responses of the offspring. Earlier studies demonstrated that prenatal stress has been associated with development of abnormal behaviour in later life of the offspring such as attention deficit hyperactivity disorder (ADHD) (Rodriguez and Bohlin, 2005), schizophrenia (Dong *et al.*, 2015) and depression (Kingsbury *et al.*, 2016). Previous reports have demonstrated that prenatal stress was associated with alteration in the nociceptive responses of the offspring (Sandercock *et al.*, 2011; Abd Aziz *et al.*, 2013) and a report has suggested that stressful prenatal period was associated with modulation of nociceptive-neuronal network in the offspring which led to pain hypersensitivity (Chen *et al.*, 2010).

1.1.1 The effects of prenatal stress on brain

Stress during the pregnancy can have long term impacts on offspring development due to the critical periods in the development of brain circuity associated with early growth (Weinstock, 2005). Stress during pregnancy can also lead to increase glucocorticoid in the blood of the dams and the foetus, and may contribute to increased oxidant level and alterations of the structure and function of the developing brain (Mennes *et al.*, 2009; Buss *et al.*, 2010; Sahu *et al.*, 2012).

Previous study has shown that prenatal stress was associated with decreased numerical density of neurons and altered neurotransmitter systems in the hippocampus (Dyuzhikova *et al.*, 2012). The alteration might be due to vulnerability of the brain to oxidative injury as it has modest antioxidant defences (Halliwell, 2006). As a result, the brain produces a large amount of free radical by-products, consequently lead to an imbalance between production and secretion of free radicals and antioxidants that result in oxidative stress (Ha *et al.*, 1998). A significant decrease in number of neurons and an increment in production of reactive oxygen species as well as expression of neuronal nitric oxide synthase were found in prenatally stressed rat hippocampus (Zhu *et al.*, 2004). The study demonstrates that prenatal stress is associated with oxidative stress and neurons injury which leads to neuronal loss in the brain of offspring (Zhu *et al.*, 2004).

1.1.2 Thalamus and pain

Previous findings have shown the effects of prenatal stress on cerebral cortex (Charil *et al.*, 2010), cerebellum (Maur *et al.*, 2012), hippocampus (Walf and Frye, 2012) and amygdala (Zohar and Weinstock, 2011) but no report has yet been done in the thalamus. Thalamus is regarded as an entrance to the cerebral cortex. Almost all somatosensory inputs converge on to the thalamus including the nociceptive inputs. The thalamus is a crucial relay station for transmitting nociceptive information to the cerebral cortex. Somatic sensation such as nociception or reactivity to tissue damaging stimuli is conveyed via nociceptors to the spinal cord, which later transferred by ascending tracts to the thalamus and cerebral cortex, where pain is recognised. In 1911, Head and Holmes proposed the thalamus as the important organ of the affective side of our sensation, especially pain. The thalamus is a key to pain sensation and induced pain in chronic pain conditions. A few studies indicated the

involvement of both the lateral and medial thalamus in pain processing as bilateral lesion of one of these areas would change pain behaviours (Saadé *et al.*, 1999; Wang *et al.*, 2007).

Pain receptors or nociceptors will be stimulated by noxious stimuli. During inflammation, cells are damaged and several chemicals are released including histamine, bradykinin, serotonin, potassium and prostaglandins which may sensitise nociceptors and produce hyperalgesia and allodynia. Prolonged discharge of nociceptors (C-fibres) results in release of glutamate which acts on N-methyl-D-aspartate (NMDA) receptors. Activation of NMDA receptors results in increased cell response to pain stimuli resulting in central sensitisation. There is growing evidence to show that NMDA receptors are associated with generation and maintenance of central sensitisation during pain conditions. NMDA receptors also mediate peripheral sensitisation and visceral pain.

Moreover, thalamic NMDA receptors contribute to the development and maintenance of hyperalgesia in the inflammatory pain model (Kolhekar *et al.*, 1997). Study conducted by Reyes *et al.* (2012) demonstrated that the activation of neuronal NMDA receptors induced the release of superoxide mediated oxidative stress in neighbouring neuron and astrocytes. The mechanism for the increase in oxidative stress markers is might be due to calcium dysregulation as a result of NMDA receptors hyperactivity which lead to neurotoxicity (Brittain *et al.*, 2012). However, the exact mechanisms of NMDA receptors in the thalamus offspring exposed to prenatal stress leading to alteration of nociceptive behaviour in the offspring are not yet clearly understood. Available evidence shows that prenatal stress are more susceptible to oxidative stress due to higher levels of oxidants or lower antioxidants.

Therefore, it has been postulated that antioxidant supplementation in such individuals may be beneficial.

1.1.3 Therapeutic potential of Tualang honey

A type of rainforest honey known as Tualang honey contains the strongest antioxidant and act as a free radical scavenger. It had variety kinds of phytochemicals which serves as sources of dietary and potential health benefits. Tualang honey can be used as an alternative treatment because of its antioxidant property. Prior studies also found that co-administration of Tualang honey with other therapeutic agents may be effective in minimising the side effects of synthetic drugs (Erejuwa *et al.*, 2012).

Generally, Tualang honey is a natural product consisting of variable compositions. These variances based on floral sources, geographical origin, total phenolic content, water proportion and colour (Frankel *et al.*, 1998; Mohamed *et al.*, 2010). Previously, it has been extensively studied in the treatment of various medical conditions and has been found to have therapeutic activities including antibacterial (Nasir *et al.*, 2010), anti-inflammatory (Bashkaran *et al.*, 2011), antiproliferative (Ghashm *et al.*, 2010), anticancer (Fauzi *et al.*, 2011), antidiabetic and antioxidant properties (Erejuwa *et al.*, 2010).

Tualang honey was chosen in this study as the previous studies demonstrated that Tualang honey seems to be more effective than other honey because it contains higher flavonoids and phenolic content (Khalil *et al.*, 2011; Kishore *et al.*, 2011). It contains phenolic compounds with good colour intensity which has good antioxidant activity (Mohamed *et al.*, 2010). Antioxidant protective effect of Tualang honey able to modulate anxiety-like behaviour (Al-Rahbi *et al.*, 2014c), improves brain

morphology (Othman *et al.*, 2015) and reduce brain oxidative stress (Erejuwa *et al.*, 2010). The antioxidant property of Tualang honey would be beneficial to prevent alteration of the brain structure and function that might contribute to development of pathological pain following prenatal stress.

1.2 Justification of the study

Although, several studies have demonstrated that prenatal stress is associated with development of oxidative stress and activation of NMDA receptors in the central nervous system, the exact mechanisms of maternal stress leading to alteration of nociceptive responses in the offspring are not yet clearly understood. Whether prenatal stress may affect the level of oxidative stress markers, morphology and NMDA receptors in the thalamus need to be investigated. In addition, it is not known whether there are protective effects of Tualang honey administration during prenatal stress on the alteration in the level of oxidative stress markers, morphology and NMDA receptors in the thalamus of male rat offspring.

1.3 Research objectives

1.3.1 General objectives

To investigate the effects of prenatal stress on nociceptive behaviour, level of oxidative stress parameters, morphological changes and NMDA receptors in the thalamus of prenatally stressed male rats offspring and to determine the protective effects of Tualang honey administration during prenatal stress on these parameters.

1.3.2 Specific Objectives

1. To determine the effects of prenatal stress on nociceptive behaviour, oxidative stress parameters, morphology and level of NMDA receptors in the thalamus of male rats offspring.
2. To determine the effects of Tualang honey administration to the pregnant dams on nociceptive behaviour of prenatally stressed male rats offspring.
3. To determine the effects of Tualang honey administration to the pregnant dams on oxidative stress parameters in the thalamus of prenatally stressed male rats offspring.
4. To determine the effects of Tualang honey administration to the pregnant dams on thalamus morphology of prenatally stressed male rats offspring.
5. To determine the effects of Tualang honey administration to the pregnant dams on the level of NMDA receptors in the thalamus of prenatally stressed male rats offspring.

1.4 Hypothesis of the study

1. There are significant changes in nociceptive behaviour, oxidative stress parameters, morphology and level of NMDA receptors in the thalamus of prenatally stressed male rats offspring.
2. There is a significant change in nociceptive behaviour of the prenatally stressed male rats offspring following Tualang honey administration to the pregnant dams.
3. There is a significant change in the level of oxidative stress parameters of prenatally stressed male rats offspring following Tualang honey administration to the pregnant dams.

4. There is a significant change in thalamus morphology of prenatally stressed male rats offspring following Tualang honey administration to the pregnant dams.
5. There is a significant change in the level of NMDA receptors of prenatally stressed male rats offspring following Tualang honey administration to the pregnant dams.

1.5 Research questions

1. Are there any changes in nociceptive behaviour, oxidative stress parameters, morphology and level of NMDA receptors in the thalamus of prenatally stressed male rats offspring?
2. Is there any change in nociceptive behaviour of the prenatally stressed male rats offspring following Tualang honey administration to the pregnant dams?
3. Is there any change in the level of oxidative stress parameters of prenatally stressed male rats offspring following Tualang honey administration to the pregnant dams?
4. Is there any change in thalamus morphology of prenatally stressed male rats offspring following Tualang honey administration to the pregnant dams?
5. Is there any change in the level of NMDA receptors of prenatally stressed male rats offspring following Tualang honey administration to the pregnant dams?

CHAPTER 2

LITERATURE REVIEW

2.1 Pregnancy

Pregnancy occurs when a fertilised egg (zygote) is implanted in a woman's uterus (Jones, 2010). The length of gestation averages 266 days to 280 days, or 38 to 40 weeks after ovulation (Yates, 2010). Meanwhile, in rats, the duration of pregnancy is about 21 to 22 days (Amugongo and Hlusko, 2014). Pregnancy is an experience that changes various prospect and has important consequences for healthy life and social roles in women (Striegel-Moore *et al.*, 1996; Yali and Lobel, 2002).

2.1.1 Physiological changes during pregnancy

During pregnancy, the pregnant mother undergoes a lot of physiological and homeostatic mechanisms which are necessary to ensure proper foetal development. These changes begin after conception and affect every system in the body (Lockitch, 1997). Early changes during pregnancy occur due to increased metabolic needs secondary to development and growth of foetus, uterus and placenta, and high level of hormones especially oestrogen and progesterone. The physiological changes include the changes in cardiovascular and systemic circulation, reproductive organ, urinary tract, respiratory tract and endocrine system (Campbell and Lees, 2000). However, starting from mid pregnancy onwards, the changes are partly contributed by mechanical pressure from the growing uterus (Ciliberto and Marx, 1998).

2.1.2 Endocrinological changes of pregnancy

During pregnancy, there are a lot of physiological changes that involves the endocrine system of the mother and her foetus (Kumar and Magon, 2012). The endocrine regulation of human foetal growth involves interaction between the foetus, placenta and mother (Murphy *et al.*, 2006). Steroid hormone released by the endocrine system is important to regulate physiological events that are essential for maintaining pregnancy and foetal development (Pepe and Albrecht, 1995).

All steroid hormones are derived from cholesterol. They are secreted by adrenal cortex, ovary and placenta during pregnancy. The adrenal glands secrete a lot of steroid hormones such as mineralocorticoid for sodium regulation and also androgens for regulation of sexual function and reproduction (Venning, 1946). Meanwhile, the ovaries produce the hormones necessary for the growth and development of primary and secondary reproductive organs and structures, whereas, the placenta plays an important role in synthesising and secreting peptides and steroid hormones that regulate hormonogenesis by the endocrine glands in the mother and the foetus (Tal *et al.*, 2000).

The release of hormones is largely controlled by the foetal-placental unit (FPU). Many endocrine and metabolic changes that occur during pregnancy can be directly associated to hormonal signals originating from the FPU (Tal *et al.*, 2000). The uterus in pregnant woman is a home for the growing foetus and any type of stress on the mother may contribute to adverse effect on pregnancy outcomes (Mulder *et al.*, 2002) or have a long-term effect on brain and behaviour disorders in offspring (Weinstock, 2008).

In pregnancy, the level of cortisol (stress hormone) is higher than non-pregnant level by the third trimester (Jung *et al.*, 2011) due to oestrogen stimulation of corticosteroid-binding globulin synthesis (Demey-Ponsart *et al.*, 1982; Qureshi *et al.*, 2007). Moreover, the placenta releases a high number of corticotrophin-releasing hormones (CRH) into the mother's bloodstream during the last trimesters of pregnancy (Hillhouse *et al.*, 1993; Petraglia *et al.*, 1993; Reis *et al.*, 1999). During stress, regulation of the maternal hypothalamic-pituitary-adrenal (HPA) axis undergoes significant changes as described in section 2.2.1 (a).

2.2 Stress

Stress can be described as negative "emotional experience accompanied by predictable biochemical, physiological and behavioural changes". Stress also refers to situations that tend to disturb the stability between living organisms and their surroundings. In daily life there are various stressful situations such as work pressure, emotional and psychosocial stress, as well as physical stress due to trauma, surgery and numerous medical disorders (Baum, 1990). In animal studies, there are various models of stress that can be used to investigate pathophysiology of acute stress disorders, depression and anxiety.

Chronic stress is an individual response to emotional stress experienced over a long period of time. Several studies suggest that chronic stress has shown a relationship between life events and psychological adjustment (Latendresse, 2009). It was also reported that chronic stress was associated with increased incidence of preterm delivery (Latendresse, 2009).

In contrary to chronic, acute stress refers to sudden stressful changes in an individual's situation or conditions. In animal studies, acute stress may be acquired

by releasing sudden loud noise or by keeping the animals in a situation that is restricted for a certain period. The study conducted by Sobrian *et al.* (1997) demonstrated that the exposure of pregnant animals to physiological stimulation caused by the noise results in alterations of development and immune system in the offspring.

2.2.1 Prenatal stress

Prenatal stress refers to the stress experienced by a mother during her pregnancy, usually due to stressful life events or environmental difficulties (Mulder *et al.*, 2002; Markham and Koenig, 2011). Prenatal stress can be chronic, associated with ongoing events in a woman's life, or acute, related to sudden changes in a women's daily routine or environment.

2.2.1 (a) Endocrinological changes during prenatal stress

The pregnant women who are exposed to stress may develop numerous endocrine changes, including increased catecholamine and activation of HPA axis. During stress, catecholamine such as epinephrine and norepinephrine will be released by the sympathetic-adrenal medullary system into the blood. These hormones are important in a "fight or flight" response, facilitating several physiological changes such as increased in heart rate, blood pressure, mental activity, mobilization of energy stores and cellular metabolism and a decrease in blood flow to organs that are not needed for rapid activity (Guyton, 1981).

Meanwhile, the activation of maternal HPA axis will stimulate glucocorticoid secretion. The placenta forms a structural and biochemical barrier to many of these maternal factors, although some will still enter the foetal circulation. There may also

be indirect effects on the foetus through modification of placental function. As an example, maternal glucocorticoid may stimulate the production of CRH by the placenta which in turn will activate the foetal HPA axis (Challis *et al.,* 2000). Glucocorticoid will further stimulate the placenta to release more CRH. Placental CRH, on the other hand, will stimulate the pituitary and adrenal glands that lead to higher level of glucocorticoid in the maternal blood (Figure 2.1).

The foetus is partially protected from a high maternal glucocorticoids (stress hormones) level by the action of the placental enzyme 11-β-hydroxysteroid dehydrogenase type-2 (11βHSD2) (Duthie and Reynolds, 2013). This enzyme protects the foetus from excessive exposure to glucocorticoid by catalysing the conversion of cortisol to cortisone (Murphy *et al.*, 1974; Bernal *et al.*, 1980; Benediktsson *et al.*, 1997).

Almost 80-90% of cortisol is metabolised by the placenta during gestation, but, high cortisol level can still reach the foetus (Gitau *et al.*, 2004) and the 'boundary' may be weakened following stressful events e.g. maternal anxiety (Glover *et al.*, 2009; Ponder *et al.*, 2011), infection (Johnstone *et al.*, 2005) and inflammation (Kossintseva *et al.*, 2006) enabling more cortisol transfer from mother to foetus. Exposure to high levels of cortisol may affect the development of foetal structure and therefore influence the physical, cognitive of behavioural effect in the adult offspring (Groothuis *et al.*, 2005; Kapoor *et al.*, 2006).

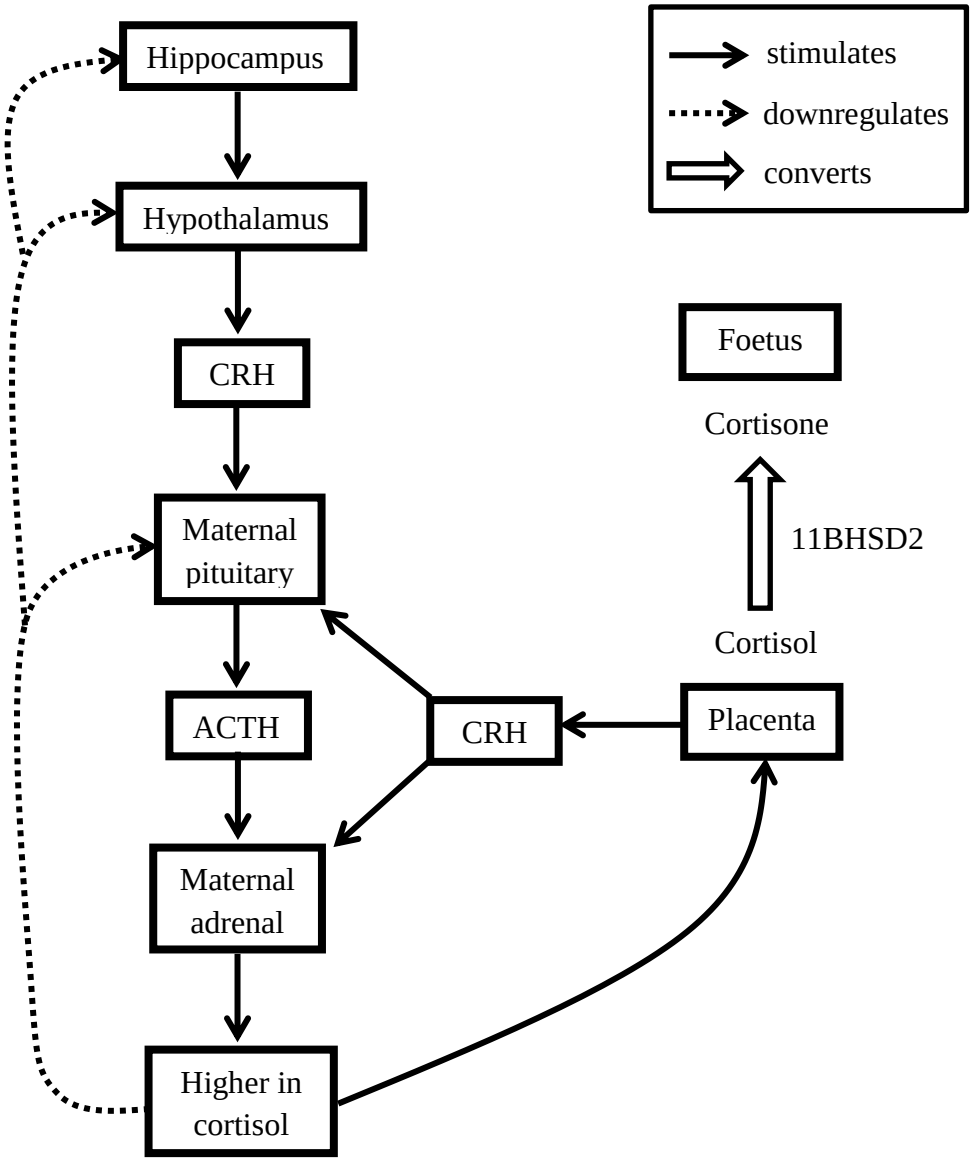

Figure 2.1 HPA axis in pregnancy. During pregnancy, maternal pituitary and adrenal is stimulated by placental CRH, which leads to an increase cortisol production. High cortisol level can stimulate the production of placental CRH. Passage of cortisol across the placenta is partially inhibited by placental 11βHSD2. The enzyme will convert cortisol to inactive metabolite (cortisone). Excessive exposure of glucocorticoid has adverse outcome to the growing foetus. [Modified from Duthie and Reynolds (2013)]. HPA: hypothalamic-pituitary-adrenal axis, CRH: corticotrophin-releasing hormone, ACTH: Adrenocorticotropic hormone, 11βHSD2: 11-β-hydroxysteroid dehydrogenase type-2.

2.2.2 Animal stress model

To assess the effects of prenatal stress on the offspring, various animal models have been used. The type of stress used depends on the objectives and parameters of the research. Several types of stressors are widely used which include neonatal isolation stress, noise-induced stress, low temperature-induced stress and physical restraint stress.

2.2.2 (a) Neonatal isolation stress

Neonatal isolation or maternal separation is an early-life stressful event that contributes to long-term effects on neural and behavioural development in the adult offspring (Francis and Meaney, 1999). During the neonatal isolation procedure, a new born offspring is removed from the cage after birth and is put in another cage for one hour in a room located separately from the animal facility. The white sound is played in the background to cover the sounds of other pups. After 1 hour, the litter is placed back in the cages with their dams (Kosten *et al.*, 2000; Kosten and Ambrosio, 2002). Separation procedure is performed once a day for 8 days. This model has been widely used to demonstrate the effect of early lifetime stress on anxiety like behaviours in the offspring (Kosten and Kehoe, 2005; Lai *et al.*, 2008; Maniam and Morris, 2010; Babygirija *et al.*, 2012).

2.2.2 (b) Noise-induced stress

Human are often exposed to noise that may be harmful in the daily life. In laboratory animals, noise stress can be induced by using loudspeakers (15 W) connected to a noise generator (0-26 kHz) placed 30 cm above the cage. Animals can be exposed to

the noise protocol either acutely or repeatedly depends on the level of noise (4 hours/day/15 days) (Manikandan and Devi, 2005; Ravindran *et al.*, 2005).

2.2.2 (c) Low temperature-induced stress

Changes in body temperature result in stressful responses because of the activation of the thermoregulatory centre and HPA axis (Kvetnansky *et al.*, 1971). The sudden reduction in temperature by using cold water such as immersed the animals in cold water (15-18 °C for 15-30 min) or place the animals (in their home cages) in cold or isolated environment (4 °C for 15-30 min) is frequently used to induce low temperature stress in animals experiment.

2.2.2 (d) Restraint and immobilisation stress

Restraint and immobilisation stress protocols are widely used to induce stress related behaviour, biochemistry and physiological changes in animals models (Kvetnansky and Mikulaj, 1970). Basically, the restraint stress is induced by placing the animal in a cylindrical or semi-cylindrical tube with ventilation holes for 120-180 min (Padovan and Guimaraes, 2000; Campos *et al.*, 2010). Meanwhile, in the immobilisation stress protocols, animals are restrained by gentle wrapping of their upper and lower limbs with adhesive tape for 120 min (Hill *et al.*, 2009; Shansky *et al.*, 2009). The head movements are constrained by wound metal loops around the neck. This procedure can be used to induce acute or chronic stress (7-21 days). The immobilisation model produces inevitable physical and mental stress with a low rate of adaptation (Kasuga *et al.*, 1999). After the restraint or immobilisation stress, the animal reveals an increase in anxiety level in the elevated plus maze test and other tests of anxiety (Padovan and Guimaraes, 2000; Campos *et al.*, 2010). Some reports using restraint models indicate that this type of stress is sufficient to influence the

reproductive outcome in pregnant rats (Haron *et al.*, 2014) and altered nociceptive responses in the offspring (Abd Aziz *et al.*, 2013).

2.3 Effect of prenatal stress on the foetus

Apparently, the environmental effects on foetal development can influence the cognitive, behavioural and development of the offspring. Previous studies have shown that maternal exposure to stressful situations during pregnancy can have long-term effects on the neurodevelopment of the offspring.

2.3.1 Effect on cognition

Acute prenatal stress has been shown to influence the cognition of children, or the ability to think. A study was conducted to determine the children's cognitive abilities by monitoring pregnant women and their children after a severe ice storm (King and Laplante, 2005). The children reveal low cognitive and linguistic capabilities when exposed to high levels of objective prenatal stress compared to the children exposed to low levels. Another study showed correlation of prenatal stress with performance of the infant at three and eight months of age. Their findings revealed that mothers with high cortisol levels during mid-pregnancy had children with lower in cognitive tests compared to their peers. This effect was more obvious for the older children compared to the younger age group (Buitelaar *et al.*, 2003).

2.3.2 Effect on behaviour

Reports have shown that prenatal stress affects the behaviour of children (Aloisi *et al.*, 1998; Weinstock, 2001; Weinstock, 2007; Rutherford *et al.*, 2009). Children exposed to prenatal stress may have emotional problems such as abnormal attitudes and difficult to pay attention. A study was carried out on monkeys and demonstrated

that psychological disturbance during pregnancy affected the offspring behaviour (Clarke and Schneider, 1993). The offspring showed more abnormal social behaviour compared to control (Clarke and Schneider, 1993). Another study suggests that prenatal stress can affect the ability to deal with stress and can lead to behavioural disorders in conflict-inducing situations in young and adult offspring (Braastad, 1998). Previous study conducted by Takahashi *et al.* (1992) had demonstrated that prenatal stress results in increased behavioural response to stress in early life and continues into adulthood.

2.3.3 Effect on nociceptive response

The stressful prenatal events can significantly affect the development of central nervous system including the nociceptive neuronal network and this may influence the pain responses in later life of the offspring (Fitzgerald, 2005). Published data indicated an alteration of pain sensitivity to acute nociceptive stimulation in offspring exposed to prenatal stress (Kinsley *et al.*, 1988; Smythe *et al.*, 1994; Sternberg, 1999). Besides that, the nociceptive response modulation associated with prenatal stress has been observed in early postnatal ontogenesis and in adulthood. Following prenatal stress the pain sensitivity can be altered due to changes in the hormones and neuronal network in the developing brain of the offspring (Peters, 1982; Peters, 1988; Reznikov *et al.*, 2001).

There is convincing evidence that prenatal stress modulates the nociceptive sensitivity in the offspring by affecting the duration of tonic pain (Butkevich, 2002). This study suggests that prenatal stress stimulation used during critical periods of ontogeny (Kassil' *et al.*, 2000) may affect the sensitivity of the pain not only temporary, but also long-lasting pain stimulation (Smythe *et al.*, 1994). Previously,

prenatal stress has been linked to increased nociceptive response in the new-borns by demonstrating that exposure to prenatal stress elevated the perception of the new-borns to painful heel prick (Davis *et al.*, 2011).

In contrast to findings in neonatal prenatally stressed rats, a study that assessed nociception after weaning (week 8-10) found that prenatal stress was associated with increased nociceptive response to noxious stimuli or formalin injection, which showed increased sensitivity to the pain in adult offspring (Abd Aziz *et al.*, 2013). Apparently, the results show that there is a marked long-term effect of prenatal stress on nociceptive responses in the offspring. However, the exact mechanism for the alteration of nociceptive responses is not really known and further studies need to be investigated.

2.4 Pain

2.4.1 Definition of pain

In 1994, the International Association for the Study of Pain (IASP) defined pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or describe in terms of such damage". In medical situation, pain is considered a symptom of the underlying condition. The word "pain" originates from the Latin "poena" which means a fine, or penalty (Visser and Davies, 2009).

2.4.2 Nociceptor

Nociceptor is a pain receptor and it is able to detect noxious stimuli e.g. thermal, mechanical and chemical stimuli (Woolf and Ma, 2007). The nociceptors will transduce the stimuli into electrical signal before transmitted to the central nervous system. They are formed by the free nerve endings of primary afferent Aδ and C

fibres (Reddi *et al.*, 2013). The nociceptors are numerous in the periphery (skin) and it is sparse in the visceral organs.

2.4.3 Pain pathway

Pain information in the form of nerve impulses is carried from nociceptors to the spinal cord and delivered to the brain. Pain can be modified or modulated in the dorsal horn at the level of spinal cord.

There are two major pathways that transmit nociceptive signals to the brain. The first pathway is spinothalamic tract, which transmits sensory information from the periphery to the central nervous system. The first afferent neurons transfer information from peripheral sensory receptors to spinal cord. The neurons will synapse with the second order neuron at the level of spinal cord. The second order neurons will cross to the opposite site of the spinal cord and ascend to the brainstem, medulla, hypothalamus, and limbic systems before projecting to the thalamus. These brain structures have an essential role in localisation and intensity of pain, emotional pain, autonomic and affective responses to pain (Almeida *et al.*, 2004). The second order neurons will synapse with the third order neurons in the thalamus. These neurons will project and terminate in the somatosensory cortex.

Meanwhile, the second pathway of transmitting the nociceptive information is spinoreticular tract. The fibres interpret and travel up from contralateral cord to the reticular formation in the brainstem, before reaching to the thalamus, hypothalamus and further projections to the cerebral cortex. The reticular formation comprises of nerve cells in the brainstem that involved in integrating ascending and descending information between spinal cord and cerebral cortex. The areas of the reticular formation are involved with the affective or mood response to pain and also activate

brain stem areas responsible for the suppression of pain response by the descending pain pathway (Almeida *et al.*, 2004; Swenson, 2006).

2.4.4 Neurotransmitters in pain transmission

Neurotransmitters are the chemical messengers that transmit the signals throughout the synapse from one neuron to another. They stimulate the muscle fibres at the end of an axon of motor neurons. There are two types of neurotransmitters that involve in the pain transmission namely excitatory neurotransmitters and inhibitory neurotransmitters. These neurotransmitters come from different chemical classes such as endogenous opioid and non-opioid peptides, amines and amino acids. The excitatory neurotransmitters stimulate the transmission of pain impulses throughout the synaptic cleft to the nociceptive dorsal horn neurons, whereas the release of inhibitory neurotransmitters will inhibit the pain transmission process. Various types of chemical neurotransmitters involved in pain transmission are listed in Table 2.1.

2.4.5 Modulation of pain

There are two types of modulation at the level of the spinal cord; segmental modulation and descending regulation. Segmental modulation of nociceptive afferent impulses is by postsynaptic inhibitory mechanisms that act on second order neurons and involve inhibitory neurotransmitters such as gamma-aminobutyric acid (GABA) or glycine (Game and Lodge, 1975; Duggan, 1982). It has been suggested that the circuits in the central nervous system can modulate pain information. The gate control theory and the descending inhibition are one of such a circuit.

Table 2.1 Chemical neurotransmitters and neuromodulators involved in pain transmission (Steeds, 2009).

Class	Neurotransmitters	
	Excitatory	Inhibitory
Amines	• Adenosine triphosphate (ATP)	• Noradrenaline • 5-hydroxytryptamine or serotonin (5-HT)
Endogenous opioid peptides	• None	• Enkephalins • B-Endorphin
Non-opioid peptides	• Substances P • Calcitonin gene-related peptide	• Galanin • Cholecystokinin
Amino acids	• Glutamate	• GABA • glycine
Others	• Nitrous oxide • Bradykinin	• Cannabinoids

2.4.5 (a) Gate control theory

In 1965, Melzack and Wall has suggested the gate control theory to describe an inhibitory pain modulation process at the spinal cord level by suggesting the contribution of large nerve fibres in inhibiting the transmission of nociceptive signals. Small nerve fibres transmit nociceptive signals and large nerve fibres transmit non-nociceptive signals. Both of the fibres synapse on projection neurons which will carry the information to the brain. With pain stimulation, small nerve fibres are activated and they will block the inhibitory interneurons. The gate will open and lead to the transmission of pain impulse to the brain.

With stimulation of large fibres (Aβ fibres), the inhibitory interneurons are activated and lead to closing of the gate. When small nerve fibres (C-fibres) are stimulated without stimulation of the large fibres, the inhibitory interneurons are not activated, so there is opening of the gate that lead to pain transmission to the higher centre. On the other hand, if there is simultaneous stimulation of small and large nerve fibres, the pain transmission will be blocked because the large fibres will activate the inhibitory interneurons that lead to gate closure (Larbig, 1991).

2.4.5 (b) Descending Inhibition

The periaqueductal grey (PAG) in the midbrain is involved in descending inhibitory modulation. It contains high concentrations of opioid receptors and endogenous opioids. Descending pathways project to the dorsal horn and inhibit pain transmission. These pathways are monoaminergic, utilising noradrenaline and serotonin as neurotransmitters.

The PAG receives input from the hypothalamus, amygdala and the cortex. Its neurons project to the medulla. The fibres then descend downwards to activate the inhibitory interneurons in the spinal cord, which can inhibit the incoming pain transmission. Another finding also indicated that PAG excited neurons in the locus ceruleus (cluster of neurons on the pons) which then excite the noradrenergic efferent in the dorsolateral tegmental nucleus (Samuels and Szabadi, 2008) which then inhibit pain signals.

2.4.6 Sensitisation of pain

Sensitisation occurs when the excitability of neurons increases (hypersensitivity) and they become more responsive to stimuli or sensory inputs. Typically, a strong stimulus is required to stimulate the nociceptors. However, following sensitisation, they are stimulated by innocuous stimuli and their activation will produce pathological pain and is a reflection of neuroplasticity (Scholz and Woolf, 2002). The two types of pathological pain that occur due to neuronal sensitisation are hyperalgesia and allodynia. Hyperalgesia is a condition when there is an increase in neuron responses to noxious stimuli. Meanwhile, allodynia occurs when there is an increase in pain response to normal innocuous stimuli. Both of these conditions are contributed by two main mechanisms which are peripheral sensitisation and central sensitisation (Ji *et al.*, 2003).

2.4.6 (a) Peripheral sensitisation

The IASP describes peripheral sensitisation as "increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive field". In general, peripheral sensitisation is and increased sensitivity to afferent nerve stimuli. This occurs following the cell injury and neuropeptides is

produced by the nociceptors. Hence, it stimulates high threshold nociceptors in response to non-noxious stimuli which is primary hyperalgesia or primary allodynia. For example, a gentle hit to the skin prior the injury is not painful but after injury is interpreted as pain (Bolay and Moskowitz, 2002).

This can occur as a result of inflammation. The release of chemical mediators of inflammation e.g. histamine, bradykinin, acids and serotonin may either be stimulated, depolarised or sensitised (bringing the membrane potential closer to the depolarisation threshold). This affects the threshold known as peripheral sensitisation. It occurs when the chemical mediators stimulate the receptors on the nociceptive terminals and influences the protein enzyme cascade which then up-regulates the ion channels and sodium specific nociceptive channels. Subsequently, it becomes more sensitive to the chemical mediators and leads to an influx of ions which in turns stimulate the action potentials and producing pain (Bolay and Moskowitz, 2002; Staud and Smitherman, 2002).

2.4.6 (b) Central sensitisation

The IASP describes central sensitisation as "increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input" (Loeser and Treede, 2008). In general, central sensitisation refer to the conditions in which there is an increase in the function of neurons involved in nociception in the spinal cord and the brain (Smart *et al.*, 2010) resulting in hypersensitivity to stimuli (Latremoliere and Woolf, 2009), responsiveness to non-noxious stimuli (Loeser and Treede, 2008) and enhanced pain response induced by stimuli outside the area of injury.